CHARACTERIZATION OF PP2A REGULATORY B SUBUNITS

IN

FUSARIUM VERTICILLIOIDES

A Thesis

by

JOONHEE SHIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2010

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Won-Bo Shim
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Major Subject: Plant Pathology

ABSTRACT

Characterization of PP2A Regulatory B Subunits in *Fusarium verticillioides*.

(May 2010)

Joonhee Shin, B.S., Korea Advanced Institute of Science and Technology

Chair of Advisory Committee: Dr. Won-Bo Shim

Fusarium verticillioides is a pathogen of maize causing ear rot and stalk rot. The fungus also produces fumonisins, a group of mycotoxins linked to disorders in animals and humans. A cluster of genes, designated FUM genes, plays a key role in the synthesis of fumonisins. However, our understanding of the regulatory mechanism of fumonisin biosynthesis is limited. It was previously demonstrated that Cpp1, a protein phosphatase type 2A (PP2A) catalytic subunit, negatively regulates fumonisin production and is involved in cell shape maintenance. Typically, a structural A subunit, a catalytic C subunit, and a regulatory B subunit form PP2A heterotrimer complex. Significantly, there are two PP2A regulatory subunits in *F. verticillioides* genome, Ppr1 and Ppr2, which are homologous to *Saccharomyces cerevisiae* Cdc55 and Rts1, respectively. Based on preliminary data, I hypothesized that Ppr1 and Ppr2 are independently involved in the regulation of fumonisin biosynthesis and/or cell development, and to test this hypothesis I generated gene-deletion mutants of *PPR1* and *PPR2.* The *ppr1* deletion strain (Δppr1) resulted in drastic growth defect, but with increased microconidia production. The *ppr2* deletion mutant strain (Δppr2) showed

elevated fumonisin production similar to the Δ*cpp1* strain. Germinating Δppr1 conidia formed abnormally swollen cells with central septation. Δppr2 showed early hyphal branching during conidia germination. Results from this study suggest that two PP2A regulatory subunits in *F. verticillioides* carry out unique roles in regulating fumonisin biosynthesis and fungal development.

DEDICATION

To my daughter, Helen Tae-young, and my husband, Minseon Ahn

ACKNOWLEDGEMENTS

I owe my deepest gratitude to my advisor, Dr. Won-Bo Shim, for giving me considerable guidance and support throughout my graduate study which enabled me to develop an understanding of the subject. I thank all members of the Shim Lab for their assistance and friendship. I would like to extend special thanks to Drs. Jung Eun Kim and Mala Murkherjee for helpful technical advice. Finally, I would like to thank my parents and parents-in-law for their encouragement and support.

NOMENCLATURE

PP2A	Protein Phosphatase type 2A
FB_1	Fumonisin B_1
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
YEPD	Yeast Extract Peptone Dextrose
kb	kilo base pair
ABC1	An example of how wild-type genes are written in the thesis
abc1	An example of how mutated genes are written in the thesis
Δabc1	An example of how gene-deletion mutant strains are written in the thesis: Δ symbolizes ‘deletion’
Abc1	An example of how wild-type proteins are written in the thesis: first letter is uppercase, second and third letters are lowercase, and a number.

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1. INTRODUCTION

1.1 *Fusarium verticillioides* and maize diseases: Significance of the problem

Fusarium verticillioides (Sacc.) Nirenburg (teleomorph *Gibberella moniliformis* Wineland) is a fungal pathogen of maize which is found worldwide (Munkvold and Desjardins, 1997; Species Descriptions, 2007). *F. verticillioides* is known to cause maize stalk rot and ear rot (Munkvold and Desjardins, 1997; Sparks, 2009; Species Descriptions, 2007), but there are also reports demonstrating that the fungus is associated with corn seedling blight and root rot (Bacon et al., 1994; Soonthornpoct et al., 2001). These diseases will ultimately result in yield loss and in lower grain quality (El-Meleigi et al., 1983; Sparks, 2009). Furthermore, *F. verticillioides* is considered an important pathogen of maize because the fungus produces mycotoxins, notably fumonisins, fusaric acids, and fusarins, which are toxic to livestocks and humans (Marasas, 2001; McKean et al., 2006; Rheeder et al., 1992). Among these mycotoxins, fumonisins have been under the spotlight in recent years due to their high toxicity. *F. verticillioides* is also recognized as a systemic endophyte in pre-harvest maize and is known to compete on maize ears with *Aspergillus flavus*, the fungus responsible for aflatoxin contamination (Wicklow et al., 1988).

Kernel rot and ear rot often occur on individual kernels or group of kernels. The

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This thesis follows the style of Fungal Genetics and Biology.

occurrence of *F. verticillioides* increases when kernels become mature near the end of the growing season. Multiple factors, such as drought stress or host resistance, may contribute to incidence and severity of corn diseases caused by *F. verticillioides* (Chotena et al., 1980; Headrick and Pataky, 1989). Another difficulty associated with managing Fusarium kernel/ear rot and mycotoxin in maize is that *F. verticillioides*-infected corn may be symptomless (Vincelli and Parker, 2002). Thus, developing and implementing appropriate postharvest management is critical to minimizing the occurrence of ear rot and fumonisin contamination in stored maize.

1.2 *F. verticillioides* reproduction and vegetative growth

F. verticillioides is an ascomycetous filamentous fungus with asexual and sexual means of reproduction (Species Descriptions, 2007). The septated sexual spores, ascospores (Fig. 1A), are generally oval to diamond-shaped, two-celled that are produced in perithecia (Fig. 1B), sexual fruiting bodies that develops when two *F. veticillioides* of opposite mating types converge. Two types of asexual conidia are produced in *F. verticillioides*: microconidia and macroconidia. Microconidia (Fig. 1C) are small, single, non-septated and oval to club shaped spores. When aerial mycelia are formed, microconidia often form chains or small aggregates on monophialides (Fig. 1E and F), specialized structures on hyphae. Macroconidia (Fig. 1D), produced on slender phialides, are long and canoe-shaped cells, and are generally curved and tapered to a point with 3 to 5 septa. Typically, *F. verticillioides* macroconidia are difficult to generate

under laboratory conditions whereas microconidia are easy to generate on a variety of synthetic media.

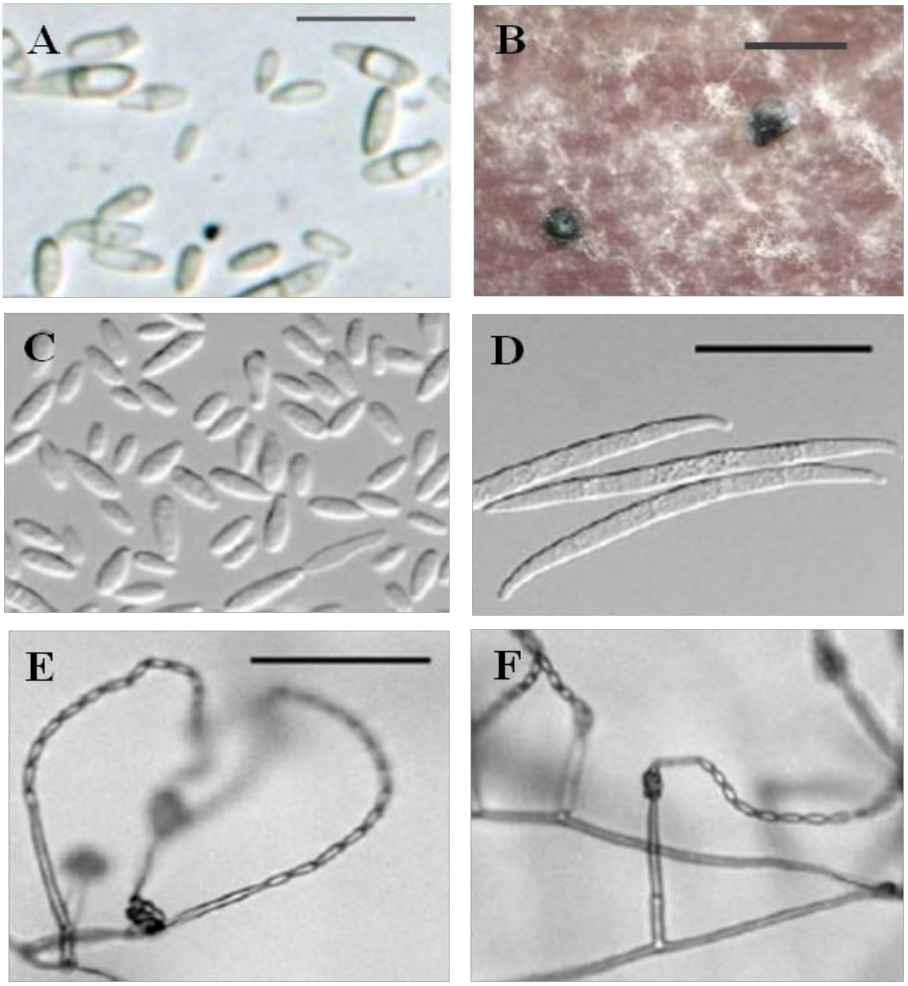

Figure 1. Sexual and asexual reproduction of *F. verticillioides*. (A) ascospores, bar=10μm; (B) perithecia, bar=500 μm; (C) microconidia, bar=25 μm; (D) macroconidia, bar=25 μm; (E -F) microconidial chains formed on monophialides, bar=50 μm. B-D were obtained from *Fusarium* Laboratory Manual (Species Descriptions, 2007).

In early developmental stages, *F. verticillioides* produces white mycelia on PDA, a commonly used laboratory medium for filamentous fungi, but violet pigmentation can often be observed in aged cultures (Species Descriptions, 2007). Significantly, pigmentation varies with media. On V8 agar, which is another conventional medium used for *Fusarium* species, *F. verticillioides* wild-type strain generates abundant aerial mycelia, but not extensive pigmentation. However, we can often observe a number of blue or blue-black sclerotia under the mycelia mat on agar media.

1.3 Fumonisins: A key group of mycotoxins produced by *F. verticillioides*

As described earlier, *F. verticillioides* produces a variety of secondary metabolites, namely mycotoxins. Mycotoxins are toxins produced by fungi that have detrimental health effects on humans and animals. One of the key mycotoxins produced by *F. verticillioides* is fumonisins. Fumonisins are a group of mycotoxins produced primarily by *F. verticillioides* and *F. proliferatum* in maize (Frisvad et al., 2006). Fumonisins are polyketide-derived mycotoxins which are structurally similar to sphingolipid intermediates (Wang et al., 1991). Fumonisins are known to inhibit ceramide synthase resulting in disruption of sphingolipid metabolism (Marasas et al., 2004). This can lead to various cellular malfunctions resulting in defective growth and in the blocking of cell-to-cell communications. Fumonisins are produced in maize kernels or maize-based products, and when consumed by humans or animals can cause harmful health effects. Equine leukoencephalomalacia (ELEM) is a well-known neurotoxic

disease in horses, donkeys, or mules caused by fumonisins. Numerous cases of ELEM were reported in the corn-growing regions in the United States in the early 20th century, and it was later determined that ELEM was directly linked to fumonisin-contaminated feeds (Marasas et al., 1984). Fumonisins are also associated with liver damage in pigs and rodents, abnormal development of bone in poultry, and neural tube defects in humans. Moreover, the link between fumonisins and human cancer has been intensively studied since the reports describing the association of human esophageal cancer to fumonisin-contaminated corn (Marasas et al., 2004; Rheeder et al., 1992).

F. verticillioides produces several types of fumonisins including fumonisins B_1, B_2, B_3, and B_4 (FB_1, FB_2, FB_3, and FB_4 respectively) (Munkvold and Desjardins, 1997). These fumonisins share a 19 or 20 carbon backbone with an amine, one to three hydroxyl, two methyl, and two tricarboxylic acid groups attached to the backbone (Fig. 2) (Munkvold and Desjardins, 1997). FB_1 is the major form of fumonisin found in nature, and it contains two hydroxyl groups at the C-5 and C-10 positions. The genes encoding fumonisin B_1 are clustered, which is a similar genetic mechanism when compared to other mycotoxins and secondary metabolites, such as aflatoxin, paxilline, and trichothecenes (Proctor et al., 2003). To date, we know the FUM gene cluster contains a series of fumonisin biosynthetic (*FUM*) genes in a 46-kb region on chromosome 1 (Brown et al., 2007). Proctor and colleagues (2003) were able to demonstrate that at least 15 genes in the FUM cluster are co-expressed. Among those genes, expression of *FUM1*, encoding a polyketide synthase (PKS), is critical to FB_1

synthesis. Fum1 PKS belongs to one of 15 or more PKSs in *F. verticillioides* (Brown et al., 2008). Other genes

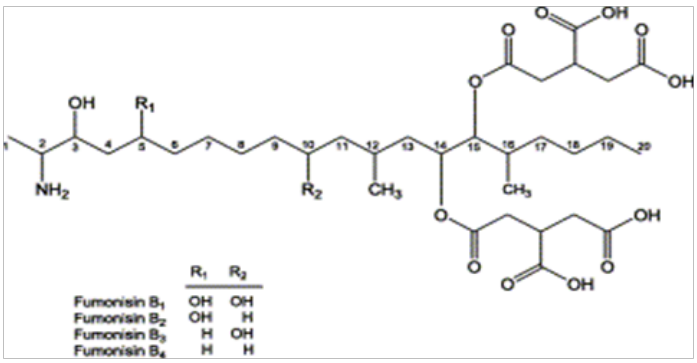

Figure 2. Chemical structure of B-series fumonisins (Proctor et al., 2003).

are predicted to be involved in the modification of the linear polyketide backbone into fumonisins (Proctor et al., 2003). However, we still do not have a clear understanding of how FB_1 biosynthesis is regulated in *F. verticillioides*, particularly how ambient nutritional conditions and host environment impact genetic regulation of *FUM* genes.

1.4 Regulation of fumonisin biosynthesis

To date, we know the fungus can produce fumonisins under certain environmental conditions, such as limited nitrogen, acidic pH, and select carbon sources (Bluhm and Woloshuk, 2005; Keller et al., 1997; Shim et al., 2003). Nitrogen limitation is known to affect FB_1 biosynthesis in a positive manner while higher concentrations of ammonium in synthetic media actually can repress FB_1 synthesis in *F. verticillioides* (Shim and Woloshuk, 1999). Published reports also indicate that low pH is a prerequisite for fumonisin biosynthesis (Keller et al., 1997). The optimal pH favoring FB_1 production turned out to be between pH 3.0 to 4.0 (Keller et al., 1997). In addition to these conditions, it seems biochemical changes inside corn kernels that occur during the development and maturation process have impact on FB_1 production: the fungus favors corn kernels that are in the latter stages of development for FB_1 production (Warfield and Gilchrist, 1999). Significantly, Blum and Woloshuck (2005) discovered that among the components of mature corn kernels, amylopectin and dextrin, the product of amylopectin hydrolysis, were key factors triggering FB_1 production when compared to other carbon sources.

We also know a number of genes identified as putative FB_1 regulatory genes. *FCC1*, which encodes a C-type cyclin in *F. verticillioides* was identified as the first regulatory gene associated with fumonisin biosynthesis (Shim and Woloshuk, 2001). *FUM1* expression was abolished and FB_1 was not detected when an *FCC1* knockout mutant was tested for FB_1 production. In addition, drastic reduction in asexual conidiation was observed in the mutant strain on corn kernels and defined liquid culture, suggesting that *FCC1* plays a role in FB_1 biosynthesis and asexual development. Subsequently, Bluhm and Woloshuk (2006) isolated *FCK1*, encoding a C-type cyclin dependent kinase, and demonstrated that the protein physically interacts with Fcc1 (Bluhm and Woloshuk, 2006). Deletion of *FCK1* resulted in phenotypes very similar to *fcc1* mutant suggesting that Fcc1 and Fck1 form the cyclin-cyclin dependent kinase complex that regulate FB_1 biosynthesis (Bluhm and Woloshuk, 2006). Interestingly, an Fck1 homolog in budding yeast controls cellular functions in response to nitrogen limitation, and thus we can hypothesize that the Fcc1/Fck1 complex is involved in nitrogen repression regulatory pathways which in turn can impact *FUM* gene expression.

ZFR1, which encodes a transcription factor with a $Zn(II)_2Cys_6$ DNA-binding motif , is another notable regulatory gene involved in fumonisin biosynthesis (Flaherty and Woloshuk, 2004). Like *FCC1*, *ZFR1* deletion resulted in severe reduction of FB_1 production. Both *ZFR1*and *FCC1* are required to FUM gene activation since constitutively expressed *ZFR1* does not restore *FUM1* expression in *fcc1*deletion strain (Flaherty and Woloshuk, 2004). Another important regulatory gene is *PAC1*, a gene encoding transcriptional regulator responsive to pH (Flaherty et al., 2003). As described

earlier, acidic pH is an important factor affecting fumonisin biosynthesis. However, in the *pac1* deletion mutant fumonisin biosynthesis was up-regulated in alkaline pH, which is a condition unfavorable to FB_1 biosynthesis in the wild type, suggesting that *PAC1* serves as a regulator of fumonisin biosynthesis (Flaherty et al., 2003). In addition to these major regulators, several additional genes, such as *GBP1* (Sagaram et al., 2006a), *GBB1* (Sagaram and Shim, 2007), *FUM21* (Brown et al., 2007), recently identified as a regulator of fumonisin biosynthesis. However, our understanding of fumonisin regulation is still limited, and it is reasonable to presume that there are many more, and complex, molecular genetic mechanisms yet to be determined (Sagaram et al., 2006b).

1.5 Role of protein phosphatase 2A (PP2A) in *F. verticillioides*

CPP1, which encodes the catalytic subunit of protein phosphatase type 2 A (PP2A) in *F. verticillioides*, was studied in an effort to understand the role of PP2A complex in the regulation of fumonisin biosynthesis. One of the key reasons that *CPP1* was chosen for molecular characterization was the fact that *CPP1* expression was up regulated in fumonisin suppression (Pirttilä et al., 2004). When Choi and Shim (2008b) investigated the phenotype of *cpp1* deletion mutant, they found that the mutant produced significantly higher level of FB_1 on corn. This result indicated that *CPP1* is a negative regulator of FB_1 biosynthesis. Furthermore, other possible functions of PP2A in *F. verticillioides* were suggested based on *cpp1* mutant phenotypes, such as macroconidia

production and multinucleated swollen cells as well as growth defects (Choi and Shim, 2008b).

PP2A is one of the major Ser/Thr protein phosphatase families with a broad substrate specificity and diverse cellular functions that are conserved throughout eukaryotes (Goldberg, 1999; Mumby and Walter, 1993). Since phosphatases are responsible for reversible dephosphorylation of proteins, PP2A plays an essential for role in signal transduction pathways associated with a wide range of cellular processes (Mayer-Jaekel and Hemmings, 1994). Particularly, PP2A is known to be involved in MAPK (mitogen-activated protein kinase) pathways (Janssens and Goris, 2001). PP2A is a heterotrimer which consists of a scaffolding A subunit, a catalytic C subunit, and a regulatory B subunit (Lechward et al., 2001; Mayer-Jaekel and Hemmings, 1994) (Fig. 3). Generally, A and C subunits, which are structurally conserved, form a stable complex (AC dimer) while the regulatory B subunit binds to this complex transiently to form PP2A heterotrimer (Fig. 4). Since studies in PP2A subunits in yeast and mammalian systems have shown evidence that the heterotrimeric PP2A forms in certain developmental stages (Goldberg, 1999; Virshup, 2000), the role of each subunit has been investigated intensively.

In *Sacharromyces cerevisiae*, there are two PP2A catalytic subunits, encoded by *PPH21* and *PPH22*, which perform redundant functions (Sneddon et al., 1990). Disrupting both genes resulted in a very small colony with a low survival rate. Deletion of a third related gene, *PPH3*, in the double-deletion mutant was determined to be lethal (Ronne et al., 1991). Double deletion of two PP2A genes in *Schizosaccharomyces*

pombe, fission yeast, resulted in lethality (Kinoshita et al., 1990). In *Neurospora crassa*, disturbing the activity of PP2A catalytic subunit by silencing gene expression or by pharmacological inhibition resulted in reduction of hyphal growth and abnormal hyphal tip formation (Yatzkan et al., 1998) .

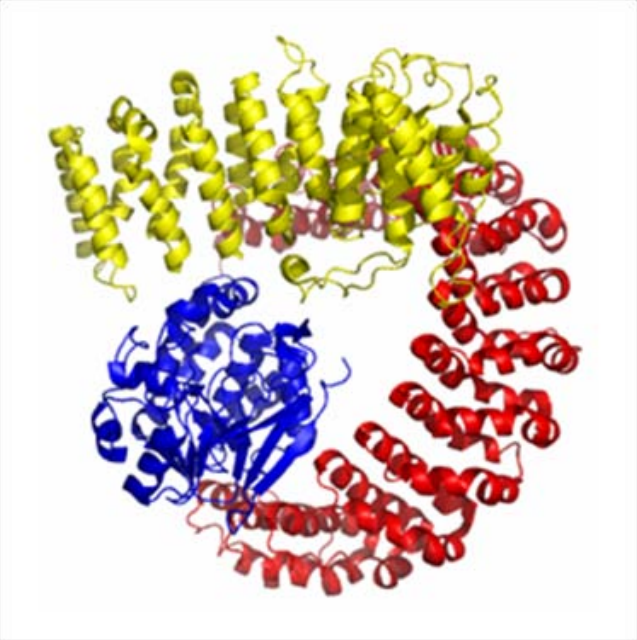

Figure 3. Structure of heterotrimeric PP2A. Red: a scaffolding A subunit, Blue: a catalytic C subunit, Yellow: a regulatory subunit. This image was obtained from Protein Bank Database Japan (Fujita).

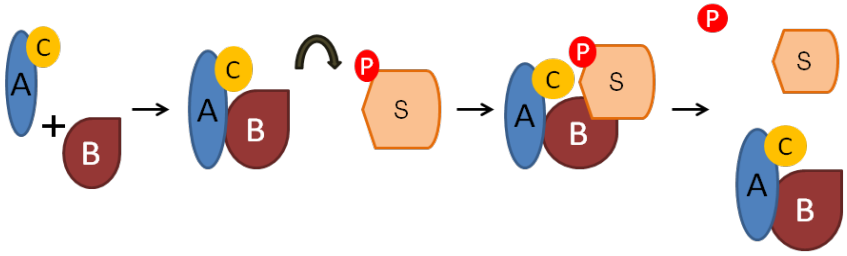

Figure 4. Proposed working model of heterotrimer PP2A holoenzyme. A: scaffolding subunit, B: regulatory subunit, C: catalytic subunit, S: substrate, P: phosphate

1.6 Role of PP2A regulatory B subunits in *F. verticillioides*

As described earlier, PP2A functions as a heterotrimer. Regulatory subunits lead the holoenzyme to specific substrates while the catalytic subunit takes action on the substrate. Therefore, it is reasonable to question how the regulatory subunits perform specific functions involved in fumonisin biosynthesis and other cellular processes in *F. verticillioides*. There are several groups of regulatory B subunits that do not share structural similarity (Janssens and Goris, 2001). But thanks to this diversity in regulatory B subunits, PP2A heterotrimer can perhaps show phosphatase activity with specific substrates (Virshup, 2000; Xu et al., 2006). In mammalian system, four major groups of PP2A regulatory subunits, which are designated B, B', B", and B"', are recognized (Lechward et al., 2001; Van Kanegan et al., 2005). In *S. cerevisiae*, two regulatory subunits of PP2A were identified: *CDC 55* encoding the regulatory B subunit of 55kDa (Healy et al., 1991) and *RTS1* encoding the regulatory B' subunit of 53 to 74kDa (McCright and Virshup, 1995; Shu et al., 1997). Gene deletion mutant strains of *cdc55* and *rts1* exhibited cold sensitivity and abnormal bud formation. What is more intriguing is that Cdc55p and Rts1p showed differential localization, indicating that different regulatory subunits could regulate specific PP2A holoenzyme functions by working with proteins located in different cellular organelles (Gentry et al., 2005). Two different regulatory subunits were also identified in *S. pombe*. When the gene encoding the PP2A regulatory B subunit $pab1^{+}$ was deleted, the mutant showed multiple abnormalities including increased temperature sensitivity, growth defect, and abnormal cytokinesis and

reproduction (Kinoshita et al., 1996). In addition, two genes encoding PP2A regulatory B' subunits in *S. pombe* were also identified and determined to play roles in mitotic cell division and interaction with calcinuerin (Tanabe et al., 2001).

One of the PP2A regulatory subunits in filamentous fungi was also characterized. PP2A regulatory subunit B in *N. crassa*, encoded by *RGB-1*, plays a role in hyphal growth and conidiation, which are essential for survival in filamentous fungi (Yatzkan and Yarden, 1999). PP2A regulatory subunit was also required for normal hyphal growth in *Sclerotinia sclerotiorum*; a study demonstrated that the PP2A regulatory subunit played an important role in sclerotial development and pathogenesis of *S. sclerotiorum* (Erental et al., 2007). However, once again we have very limited understanding of PP2A regulatory subunits in filamentous fungi and *F. verticillioides* is no exception.
Two regulatory subunits, which are designated as *PPR1* and *PPR2* (probable protein phosphatase regulatory subunit 1 and 2) were identified in the *F. verticillioides* genome database. In this study, I hypothesize that these two regulatory subunits in *F. verticillioides* play different cellular roles. To test the hypothesis, I generated gene-deletion mutants of *PPR1* and *PPR2*, designated Δppr1strain and Δppr2 strain, respectively, and investigated FB_1 production, conidiation, hyphal growth and cell morphology in Δppr1 and Δppr2.

2. MATERIALS AND METHODS

2.1 Fungal strain and culture media, conidiation, and growth assay

The wild-type *F. verticillioides* strain 7600 (also designated M-3125, Fungal Genetics Stock Center, Kansas City, KS) was stored in 30% glycerol at -80˚C. Strain 7598 (also designated m-3120) was used as the opposite mating type of strain 7600 in sexual cross experiment. *F. verticillioides* mutant strain SF41 (Choi and Shim, 2008a) which is a *FvKU70* knockout strain was used for transformation of *PPR2* deletion. Conidial suspensions for inoculum were produced by growing the fungus on V8 juice agar (200ml/L V8 juice, 3g/L CaCO3, and 20g/L agar) at 25˚C.

For genomic DNA extraction, mycelia samples were prepared by growing fungal strains in 100ml of YEPD medium (Difco, Sparks, MD) in a 250ml glass flask on a rotary shaker (125 rpm). For RNA extraction, fungal strains were inoculated in YEPD medium for 5 days, and the mycelia were harvested though filter paper (90mm Ø, Whatman). Subsequently, collected mycelia samples (0.5g) were inoculated into defined media (DM) (1g/L $NH_4H_2PO_4$, 3g/L KH_2PO_4, 2g/L $MgSO_4 \cdot 7H_2O$, 5g/L NaCl, and 40g/L Sucrose), which is known to support FB_1 production (Shim and Woloshuk, 1999).

For conidiation analysis, we prepared fungal strains grown on V8 agar medium (5mm diameter) and inoculated these on KCl agar plates (6g/L KCl and 15g/L agar). After 7 days, the conidia were harvested in 0.1% Triton X-100, and counted by using a

haemocytometer. The radius which mycelia expanded on the plate was measured to calculate the area (mm^2) where each strain grew after 7 days.

For growth assay, V8 agar blocks (5mm diameter) were inoculated on the center of PDA, V8 agar, and DM agar plates. The radius (mm) of expanding mycelia on plate was measured from day 4 to 7 with three technical replications for each strain.

2.2 *F. verticillioides* transformation

F. verticillioides protoplasts were generated following the protocol described by Shim and Woloshuk (2001) with modifications. Conidia (10^8) of wild type or SF41 mutant strain were grown in YEPD liquid media for 14 to 18 h prior to collecting mycelia. Protoplasts were prepared by suspending mycelium (1 g wet weight) in an enzyme solution (20 ml) containing lysing enzyme (10mg/ml) (Sigma, St Louis, MO), β-glucuronidase (5,200 U/ml) (Sigma), 10 mM NaH_2PO_4 (pH 5.8), 20 mM $CaCl_2$., and 1.2 M KCl. A gene disruption construct (10 μg) harboring a selectable marker, hygromycin B phosphotransferase (*HPH*) gene or geneticin resistance gene (*GEN*), was added to the protoplasts (100 μl) (Flaherty et al., 2003; Shim and Woloshuk, 2001). Transformation was performed with the aid of 40% polyethylene glycol 4000 (Sigma), and the protoplasts were regenerated in *Fusarium* regeneration agar medium, FRA, containing 1 M sucrose, 0.02% yeast extract (Difco), and 1% agar. Hygromycin B (100 μg/ml) (Calbiochem, La Jolla, CA) (Shim and Woloshuk, 2001) or Geneticin (150 μg/ml) (G418 sulfate, Gllgro®) (Sagaram et al., 2006a). Colonies that grow on the regeneration

medium amended with appropriate antibiotic were selected 3 to 5 days of incubation at 25°C.

Table 1.
Primers used in this study.

Primer	Primer sequence (5'→ 3')
PPR1-5F	AAA CTG TCT AGG CTG TCC TAT CCC
PPR1-5R	GCC GTC GTT TTA CAA GAT GAT GGA AGC TAC AAC GGC A
PPR1-3F	CAT AGC TGT TTC CTG ATG TGG CCG TTG AAG AGA CTG A
PPR1-3R	GCC GAT TTA TTG GTG TTG CCC TTG
PPR1-probF	GAC CAT ACC GGA AAC TAT CTC GCT
PPR1-probR	GCT CGT GGA TCG GTA TCG TCT TTA
PPR1-scrF	CAT AAT GGC CTG TTA GTG AGG CTG
PPR1-scrR	CGT ATA TGC TCC GCA TTG GTC TTG
M13F	TTG TAA AAC GAC GGC CAG TGA
M13R	CAG GAA ACA GCT ATG ACC ATG
YG-R	GAT GTA GGA GGG CGT GGA TAT GTC CT
HY-F	GTA TTG ACC GAT TCC TTG CGG TCC GAA
PPR2-5F	GCA TCC CTA GGG GAT CTG TTA GCG ACA
PPR2-5R	TCA CTG GCC GTC GTT TTA CAA AAG CAA CAA TAT CGA TGA TTC
PPR2-3F	CAT GGT CAT AGC TGT TTC CTG AAG CAT CGA ACT TGG ATA AGA
PPR2-3R	GCC AGA TGA TGG GTG GCT GGA ATG T
PPR2-NF	ATA ATC GAA GCG CGT TCC CTC CAA CTG GA
PPR2-NR	GGT GGA TAT GAT CGA GGT CGA GGG TGG TAA GA
PPR2-wtF	CTG CGC TTC ATT GAG AGT CAG
PPR2-scrF	CGT CTG CTG CTC CAT ACA AGC
PPR2-scrR	GTC ATA TAG CCC TGA TAA CGA TCG C
PPR2-probF	TCA CGA TTC GAT ATC TCC GCT CAC
PPR2-probR	CAA CTC GGC AAT ACC GTT GAA TCG
CPP1-probeF	CAC ATA GAC CTT CCA TTG AAG G
CPP1-probeR	TGA TCA ATC GTC CTA ATC TCA GG

2.3 Nucleic acid isolation and manipulation

Fungal genomic DNA was extracted using the OmniPrep genomic DNA extraction kit (G Biosciences, Maryland Heights, MO). Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's suggested protocol. PCR amplification of DNA was performed in a 9700 thermocycler (PE Applied Biosystems). The primers used in this study are listed in Table 1. PCR amplification of DNA (except double-joint PCR) was performed in 25 or 50 μl total volumes with Taq DNA polymerase (Promega, Madison, WI) or Expand Long Polymerase (Roche, Indianapolis, IN). The PCR conditions were 2 min of pre-denaturation at 94 °C followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 to 57 °C, and 1to 2 minutes of extension at 72 °C for Taq DNA polymerase and 68 °C for Expand Long Polymerase. Double-joint PCR was performed using Expand Long Polymerase (Roche) using the manufacturer's suggested protocol.

For Southern analysis, genomic DNA (10 μg) was digested with *Eco*RV for confirming *PPR1* gene deletion and with *Nco*I for confirming *PPR2* gene deletion. The digested DNA was subjected to electrophoresis on a 1% agarose gel and transferred onto a nylon membrane and probed with a ^{32}P labeled DNA fragment. Probes were amplified from *F. verticillioides* genomic DNA: primers PPR1-5F and PPR1-5R for *PPR1* and primers PPR2-5F and PPR2-5R for *PPR2* (Table 1).

For northern analysis, total RNA was subjected to electrophoresis in a 1.2% denaturing agarose gel containing formaldehyde and MOPS, and transferred onto a

nylon membrane. It was hybridized with ^{32}P-labelled gene-specific probes: primers PPR1-probe F and PPR1-probeR for *PPR1*, primers PPR2-probe F and PPR2 probe R for *PPR2*, and primers CPP1-probe F and CPP1-probe R for *CPP1* (Table 1). The probes used in Southern and northern hybridization experiments were ^{32}P-labelled with a Prime-It Random Primer Labeling kit (Stratagene, La Jolla, CA).

2.4 Fumonisin B_1 (FB_1) analysis

FB_1 analysis was conducted as described previously (Shim and Woloshuk, 1999) with modifications. Fungal strains were grown on cracked corn medium (B73 line; 2 g dry weight) in a 20-mL glass vial (VWR, West Chester, PA) for 14 days at room temperature (22–23 °C). To extract FB_1, we added 10 ml of acetonitrile–water (1 : 1, v/v) to each vial and stored in laboratory without agitation for 24 h. The crude extract (2 ml) was passed over equilibrated PrepSep SPE C18 columns (Fisher Scientific, Pittsburgh, PA). FB_1 concentration of samples was analyzed on a Shimatzu LC-20AT HPLC system (Shimatzu Scientific Instruments, Inc., Kyoto, Japan) equipped with an analytical Zorbax ODS column (4.6 × 150 mm) (Agilent Technologies, Santa Clara, CA) and a Shimatzu fluorescence detector (excitation 335 nm/emission 440nm). The HPLC system was operated following the protocol described by Shim and Woloshuk (1999). FB_1 was quantified by comparing HPLC peak areas with FB_1 standards (Sigma). The experiment was done with three biological replications.

2.5 Ergosterol analysis

Ergosterol was extracted from cracked corn medium (2g cracked corn kernel) incubated for 14 days after fungal inoculation. The extraction procedure was conducted following Kim and Woloshuk (2008). In detail, Fungus on cracked corn media was soaked in 10 ml of chloroform: methanol (2:1, v/v) overnight and the supernatant was collected after centrifugation. The supernatant was filtered through a Acrodisc® 13 mm syringe filter with 0.45 µm nylon membrane (Pall Life Sciences, Port Washington, NY), and directly injected into HPLC system with a 4.6 U ODS column (250 ± 4.6 mm; Alltech) and a UV detector (Shimadzu) set to monitor at 282 nm. In each sample, peak area was compared to standard curve area from HPLC-grade ergosterol (Sigma) for determining the quantities of ergosterol from each sample. The experiment was done with four biological replications.

2.6 Microscopy

Microscopic imaging was performed on Olympus BX51 microscope (Olympus America) fitted with Uplanapo objectives and an Olympus DP70 cooled charge-coupled device (CCD) digital camera. A detailed description of features used for imaging from this microscope has been given (Shaw and Upadhyay, 2005). Images of hyphal growth phenotypes were acquired using an Olympus DP70 camera and DP70-BSW software (version 01.01) and Adobe Photoshop was used for preparing publication quality prints.

A

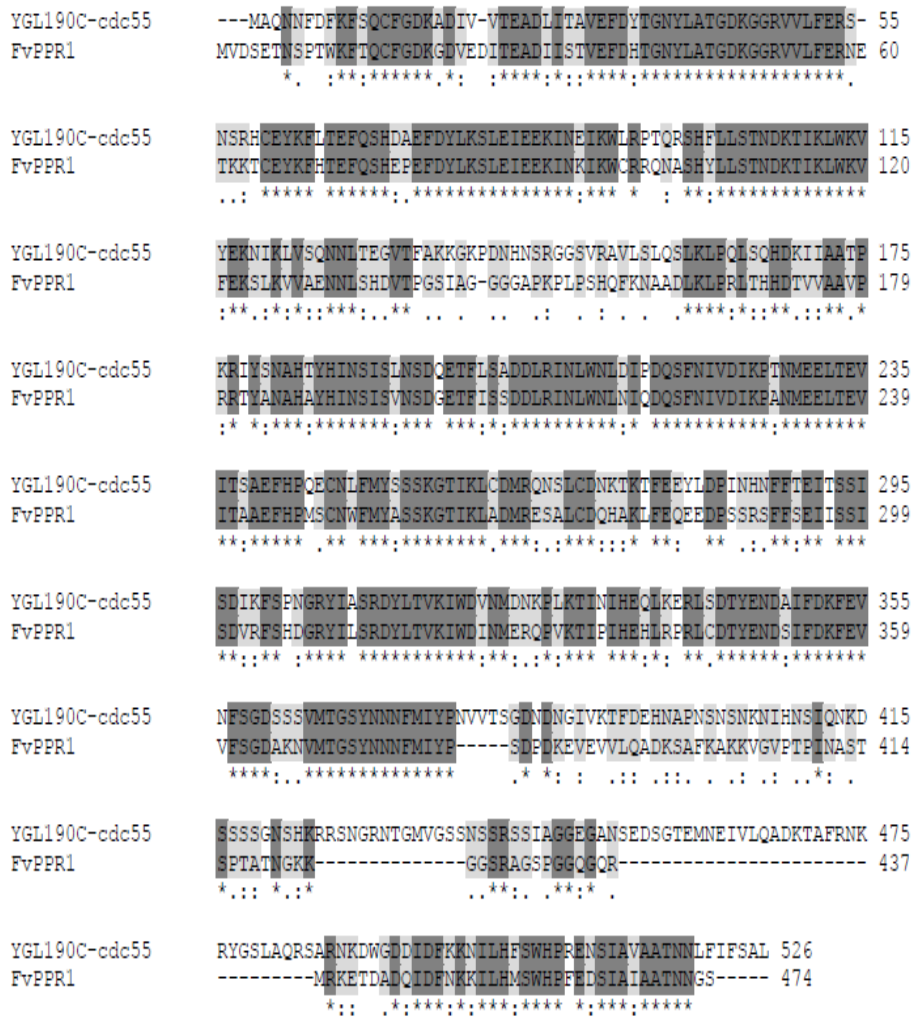

Figure 5. Protein sequence alignments. (A) Amino acid alignment of *S. cerevisiae* Cdc55 and *F. verticillioides* Ppr1 via CLUSTALW. 65% sequence identity detected between *CDC55* and *PPR1* protein sequences by WU-BLAST algorism (http://www.yeastgenome.org). (B) Amino acid alignment of *S. cerevisiae* Rts1 and *F. verticillioides* Ppr2 via CLUSTALW. 63% of conserved B56 domain identity was detected between two protein sequences by WU-BLAST.

B

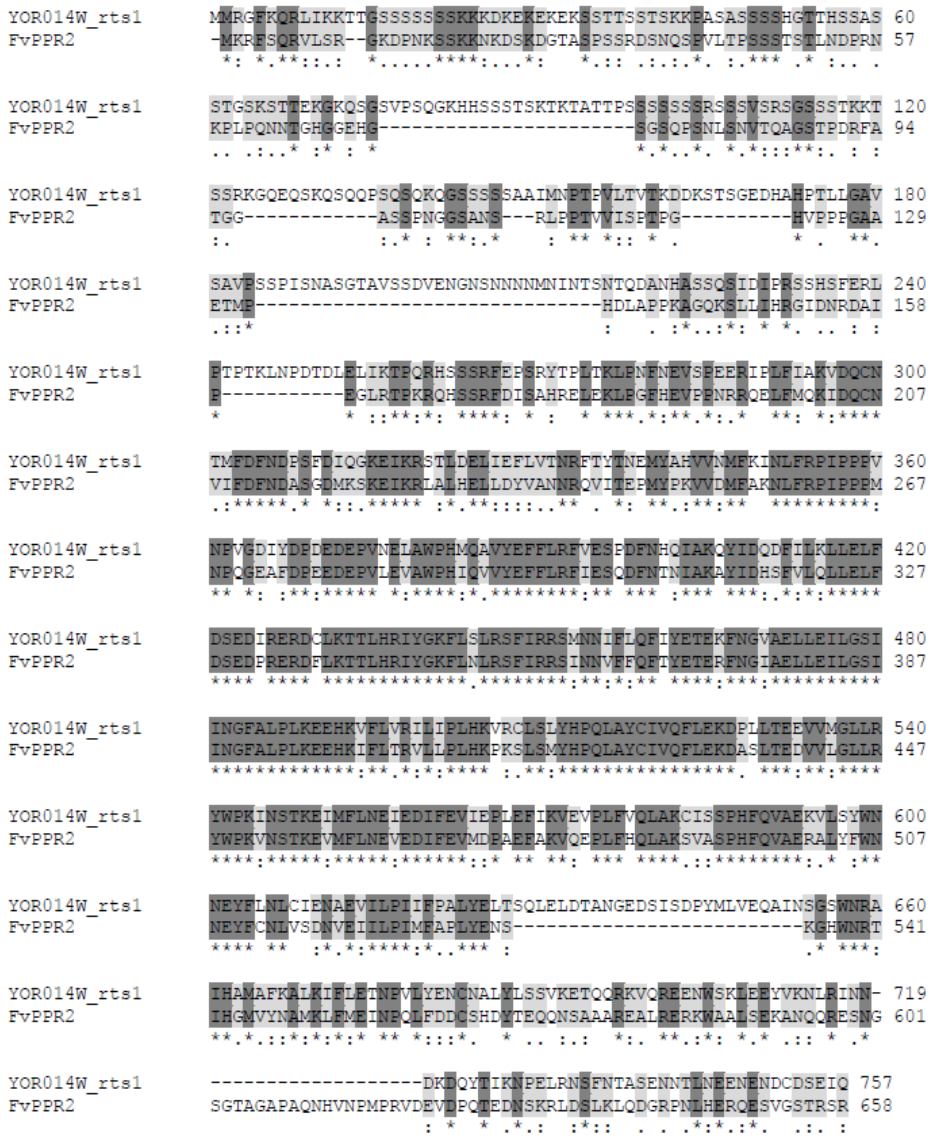

Figure 5. Continued.

3. RESULTS

3.1 *PPR1* and *PPR2* encode PP2A regulatory B subunits in *F. verticillioides*

I used protein sequences of two *S. cerevisiae* regulatory subunits, Cdc55 and Rts1 (Healy et al., 1991; Shu et al., 1997), to search the *Fusarium* group database (Broad Institute of Harvard and MIT, http://www.broad.mit.edu/annotation/fgi/), and identified the corresponding homolog of each regulatory subunit in *F. verticillioides* genome. The Cdc55 homolog was identified in supercontig 1 on chromosome 1, specifically from sequence 4599671 to 462020 (FVEG_01508). I designated this gene *PPR1*. The homolog of Rts1 was found in supercontig 5, specifically from sequence 665726 to 667909 (FVEG_04543), and I designated this gene *PPR2*. Protein sequence identity between Cdc55 and Ppr1 was 65%, and the conserved B56 domain identity between Rts1 and Ppr2 was 63% when using WU-BLAST algorithm (http://www.yeastgenome.org) (Fig. 5A and 5B).

3.2 *F. verticillioides PPR1* and *PPR2* knock-out mutants

PPR1 and *PPR2* deletion mutants were generated to determine the functions of genes encoding PP2A regulatory subunits in *F. verticillioides*. *PPR1* gene disruption construct, with 5’ and 3’ flanking regions of the target gene and hygromycin-resistance gene (*HPH*; hygromycin phosphotransferase (Punt et al., 1987)) was created using split-marker approach (You et al., 2009) (Fig. 6A). The construct was transformed into

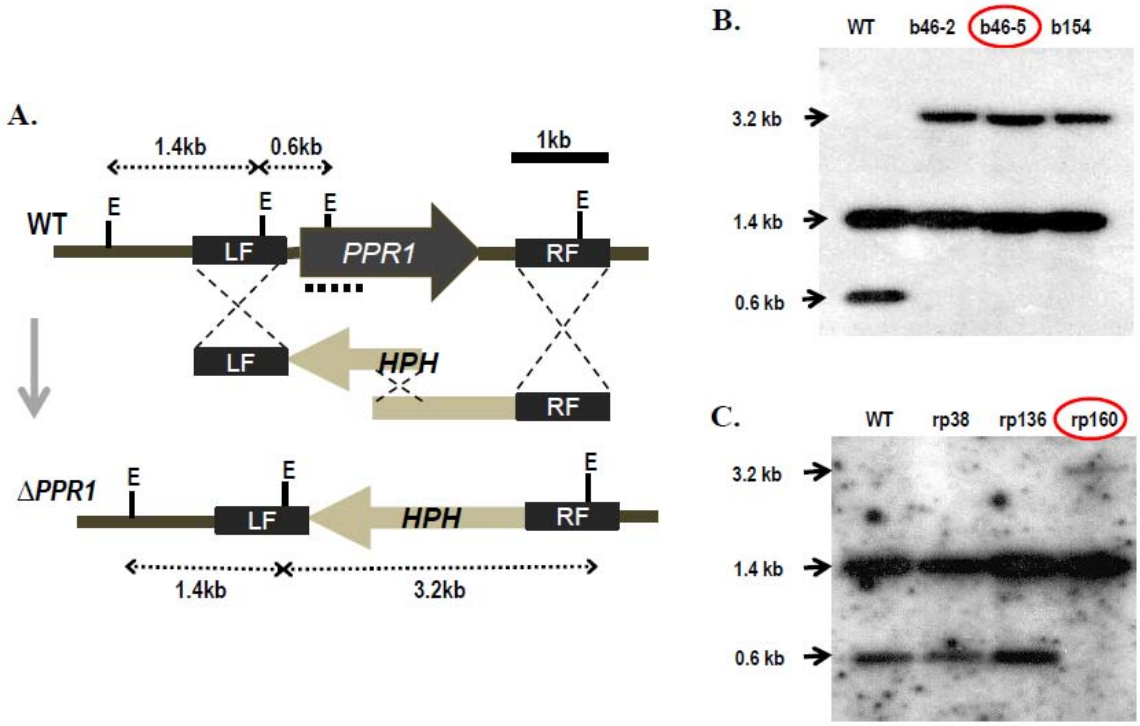

Figure 6. Targeted deletion of *PPR1* in *F. verticillioides*. (A) schematic representation of homologous gene recombination strategy resulting in gene-deletion. The dotted line underneath *PPR1* gene represents the genomic region used as a probe for northern blot. E represents the *Eco*RV restriction site. The dotted arrows indicate the size of digested DNA fragment which should appear on Southern blot; (B) Southern blots confirming *PPR1* deletions. LF region fragment was used for the probe. The red circled strain was selected to be the Δppr1 strain; (C) Southern blot confirming re-knockout of *PPR1* with the same disruption construct. The red circled strain was confirmed as *PPR1* deletion strain.

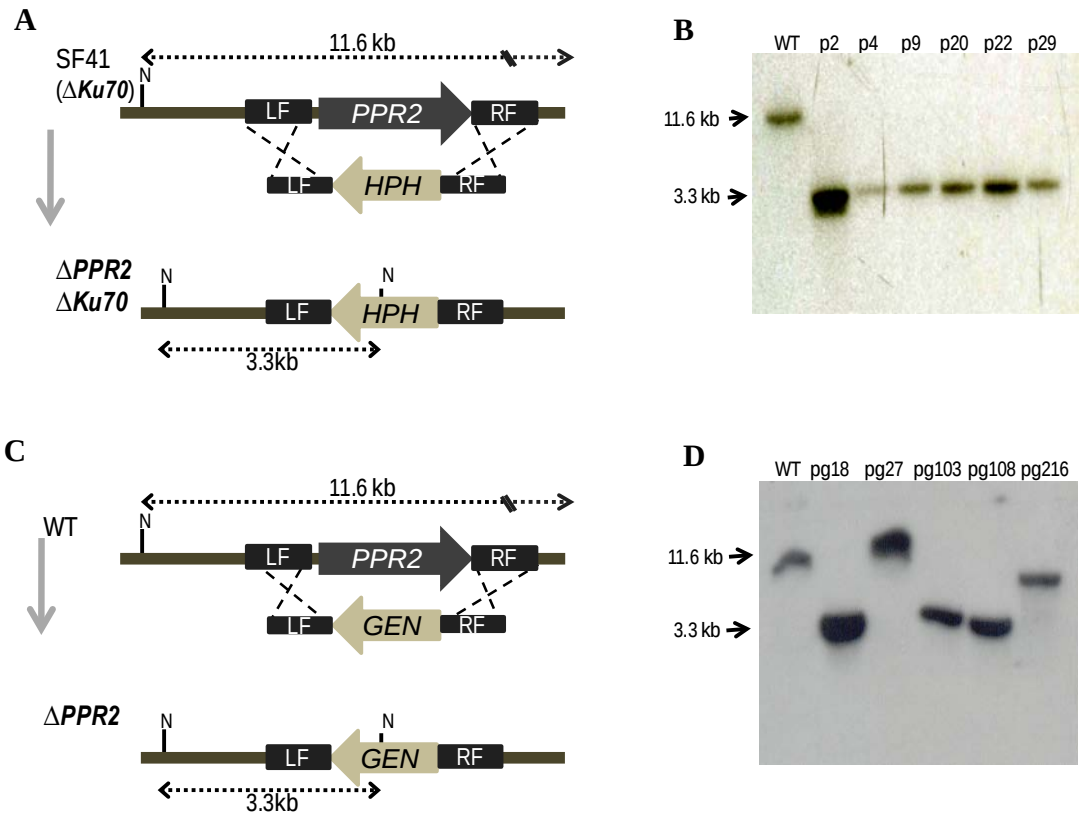

Figure 7. Targeted deletion of *PPR2* in *F. verticillioides*. (A) schematic representation of homologous gene recombination strategy resulting in gene-deletion using *HPH* as a marker in SF41 strain; The dotted line underneath gene *PPR2* represents the genomic region used as a probe for northern blot. N represents the *Nco*I restriction site. The dotted arrows indicate the size of digested DNA fragment which should appear on Southern blot; (B) Southern blots confirming *PPR2* deletion; the red circled strain was selected as Δppr2 strain. LF region fragment was used as the Southern probe; (C) Schematic representation of homologous gene recombination strategy resulting in gene deletion using GEN as a marker in wild-type strain. Same *PPR2* DNA fragment (Fig. 7A) was used as the northern blot probe. N presents the *Nco*I restriction site. The dotted arrows indicate the size of digested DNA fragment which should appear on Southern blot; (D) Southern blot confirming re-knockout of PPR2. Two re-knockout strains were appointed with red circle. LF region fragment was used as the Southern probe.

protoplasts of wild-type M3125 strain. Through PCR, I identified putative *ppr1* gene knockout mutants, and I further validated our screening with Southern analysis, using the left flanking region, LF, as the ^{32}P-labeled probe (Fig. 6A). Genomic DNA samples were digested with *Eco*RV, and I anticipated bands at 1.4 kb and 3.2 kb which would indicate that *PPR1* gene went through homologous recombination with split marker constructs, whereas wild type is expected to give bands at 1.4 kb and 0.6 kb (Fig. 6B). I isolated multiple putative mutant strains; b46 strain was designated as the Δppr1 strain and selected for further molecular characterization.

PPR2 deletion was conducted by transforming Ku70 knockout strain, SF41 (Choi and Shim, 2008a), rather than the wild-type strain in an effort to increase homologous recombination efficiency. A disruption construct (Fig. 7A) containing *HPH* gene was created with the double-joint PCR approach (Yu et al., 2004), and transformed in to SF41 protoplasts. Multiple *PPR2* deletion mutants were confirmed by Southern analysis, and these mutant strains shared identical phenotype on V8 agar (Fig. 8A). As anticipated, homologous recombination of *PPR2* locus with the disruption construct generated a 3.3kb band when LF fragment was used as a probe when genomic DNA samples were digested with *Nco*I (Fig. 7B). Among these strains, p4 strain was selected for further characterization of *ppr2* deletion, which was designated as the Δppr2 strain.

To further confirm that the mutant phenotypes observed in Δppr1 and Δppr2 are due to gene deletion in *PPR1* and *PPR2*, respectively, I conducted re-knockout experiments. The Δppr2 strain has two selectable markers, *HPH* and geneticin-resistance gene (*GEN*), since it was created in SF41, a strain that already has *GEN*. While

Δppr1strain only has a *HPH* marker, I could not perform genetic complementation of the mutation due to the difficulties associated with generating protoplasts from Δppr1 strain. For *PPR1* re-knockout, the disruption construct used earlier (Fig. 6A) was transformed into the wild-type strain. I confirmed one *PPR1* deletion mutant by Southern analysis (Fig. 6C), and this mutant showed the identical phenotype on PDA and V8 agar plate when compared to the original Δppr1 strain (Fig. 8B and 8C). For *PPR2*, I created a disruption construct following the same procedure as described earlier but with *GEN* as the selectable marker (Fig. 7C). Furthermore, the transformation was done in the wild-type M3125 strain instead of SF41 strain. Two *PPR2* deleted mutant strains were confirmed by Southern analysis (Fig. 7D), and they exhibited identical phenotype on V8 agar plate and in YEPD when compared to the Δppr2 (Fig. 8E).

3.3 *PPR2* and *CPP1* reciprocally influence transcriptional expressions

Northern analysis confirmed the deletion of genes. *PPR1* and *PPR2*, as expected, transcripts were not detected Δppr1 and Δppr2 strains, respectively (Fig. 9A and 9B). I also tested the expression of *CPP1* to see if mutations in PP2A regulatory subunits had any impact on *CPP1* gene expression. Interestingly, I found that *CPP1* expression in Δppr2 was higher than in Δppr1 and the wild type (Fig. 9C). Moreover, higher expression of *PPR2* in Δcpp1 was also observed in the previous northern blot (Fig. 9B). This result suggests that *PPR2* and *CPP1* regulate each other in a reciprocal manner.

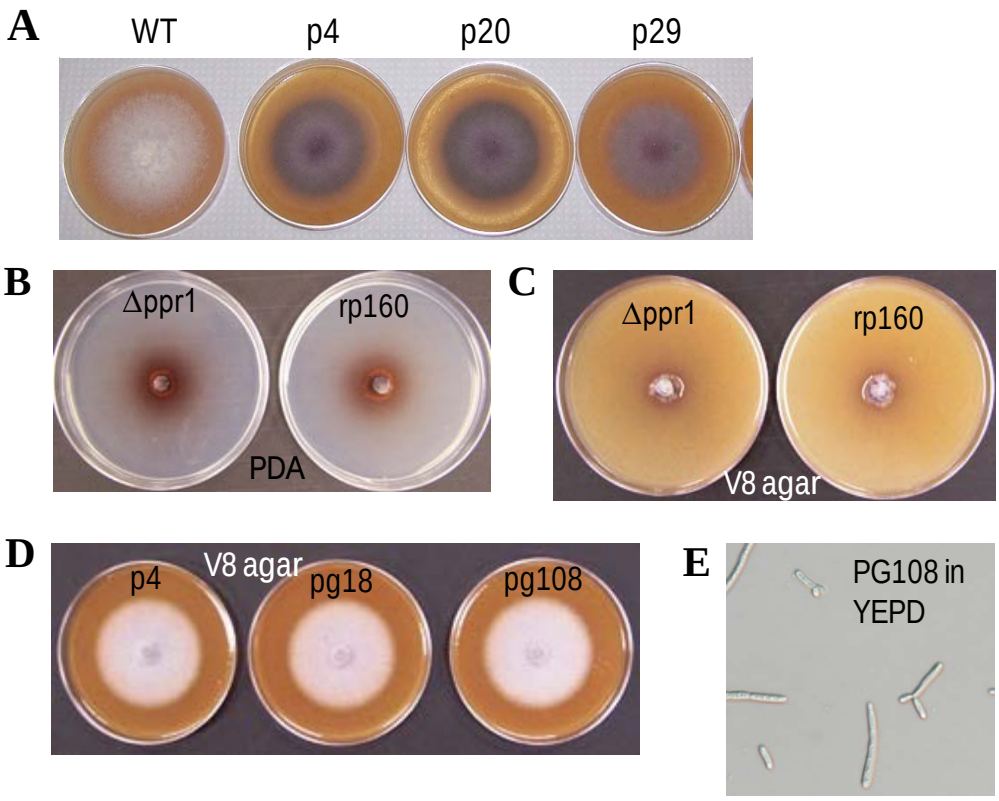

Figure 8. Confirmed *ppr1* and *ppr2* deletion mutants. (A) Growth of putative *PPR2* deletion mutant strains on V8 agar compared to growth of WT (wild-type); (B and C) Δppr1 and rp160, *PPR1* re-knock out strain, on PDA (B) and V8 agar(C) plates. (D) Δppr2 and *PPR2* re-knocked out strains, pg18 and pg108, on V8 agar plates; E, Y-shaped germinating conidia from the pg108 strain.

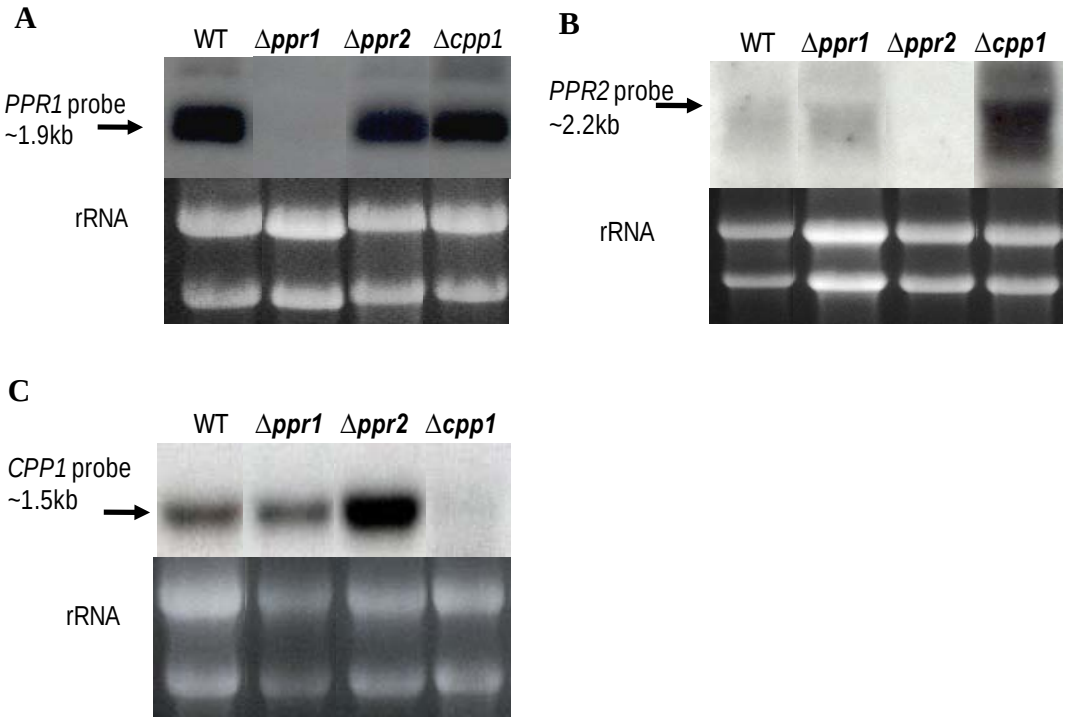

Figure 9. Transcriptional analysis. Expressions of (A) *PPR1*, (B) *PPR2*, and (C) *CPP1* were examined in wild type (WT), and deletion mutants (Δppr1, Δppr2, Δcpp1). Total RNA (12 μg) extracted from fungal cultures grown in defined medium (DL) for 7 days was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred on to a nylon membrane and hybridized with ^{32}P-labelled gene-specific probes. Target genes and transcript sizes are indicated with arrows on the left of each picture. Ribosomal RNA (rRNA) stained with ethidium bromide is shown to verify equal loading.

3.4 Δppr1 strain is severely defective in radial growth on solid media

Both *PPR1* and *PPR2* gene deletions resulted in growth deficiency in *F. verticillioides* on V8 agar, PDA, and other solid defined media. Δcpp1 was also inoculated on those solid media to compare the role of PP2A regulatory B subunits and catalytic C subunit on radial growth in *F. verticillioides*. While Δcpp1 exhibited reduced growth rate when compare to the wild type, disruption of PP2A regulatory subunits, Δppr1 and Δppr2, resulted in more drastic reduction in radial growth (Fig. 10A). The growth rate of Δppr1 drastically slowed to approximately 10 to 14% of that of the wild type (Fig. 10B). The Δppr1 strain also deposited unknown red-to-crimson pigment heavily into agar media during growth (Fig. 10A and 10C). The radial growth of Δppr2 was approximately 50% of wild type overall in all media tested (Fig.10B). On complex agar media, such as PDA and V8 agar, growth rate of Δppr2 was similar to that of Δcpp1 (Fig. 10B).

I tested the growth of these four strains (wild type, Δppr1, Δppr2, and Δcpp1) in YEPD liquid medium by measuring total fresh weight of fungal mycelia after 7-day incubations. For comparison, I also grew these strains on YEPD agar medium, and observed similar growth trend seen in other solid media. In particular, I noticed that Δppr1 strain grew extremely slow on YEPD agar (Fig. 11A). Significantly, fresh weight of Δppr1 collected from YEPD liquid culture showed no significant difference from that observed in the wild type (Fig. 11B). In liquid culture, Δppr1 developed highly compact

mycelial network throughout the culture which was quite a contract when compared to strains (Fig. 11C).

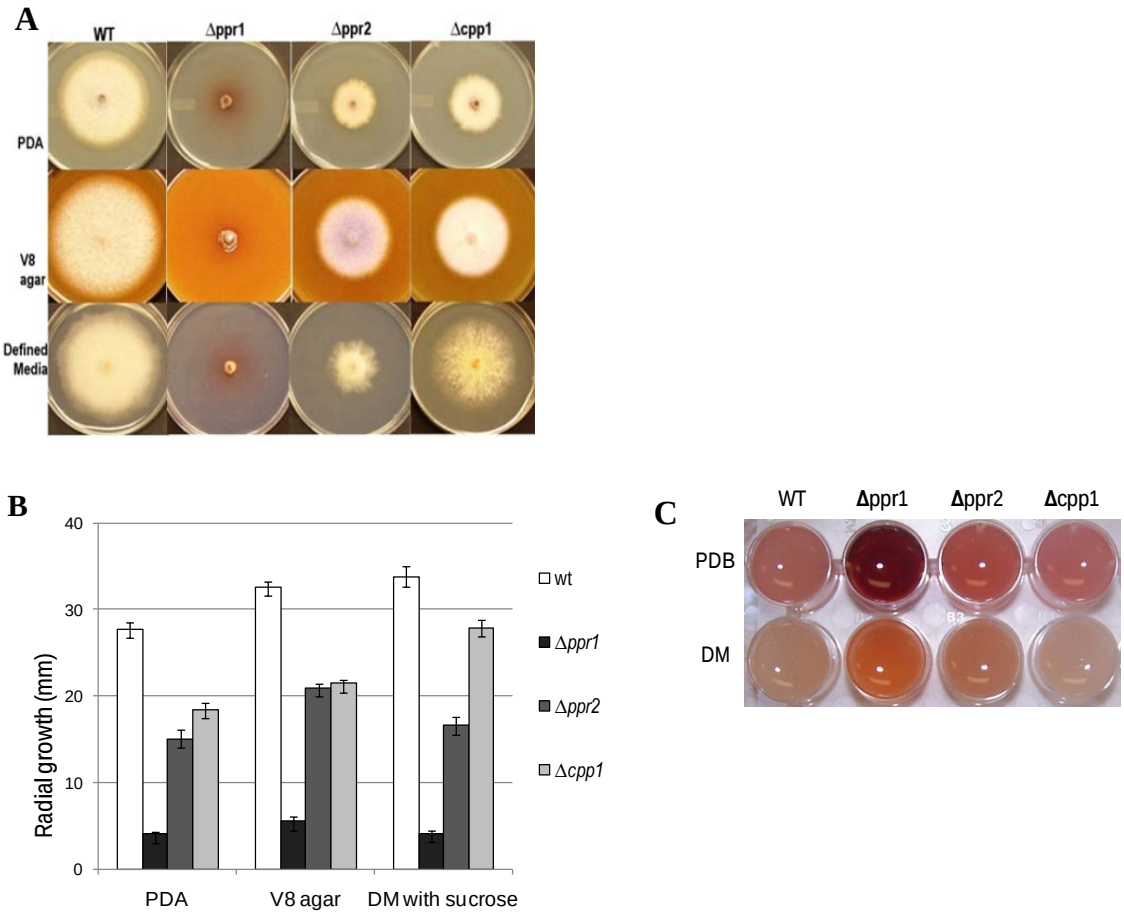

Figure 10. Colony morphology and growth of the wild type and mutant strains on various solid media; PDA, V8 agar, and Defined Medium (DM). (A) Strains were point inoculated with an agar block (0.5 cm diameter), and incubated for 7 days at 25˚C. Each strain was aligned vertically, as indicated. Media were indicated left of each row; (B) radial growth measured and presented as a bar graph. Results are means ± *sd* (standard deviation) of three biological replications. (C) Pigmentation in liquid media, PDB (potato dextrose broth) and Defined medium (DM). Δppr1 notably deposited heavy pigmentation in all media tested.

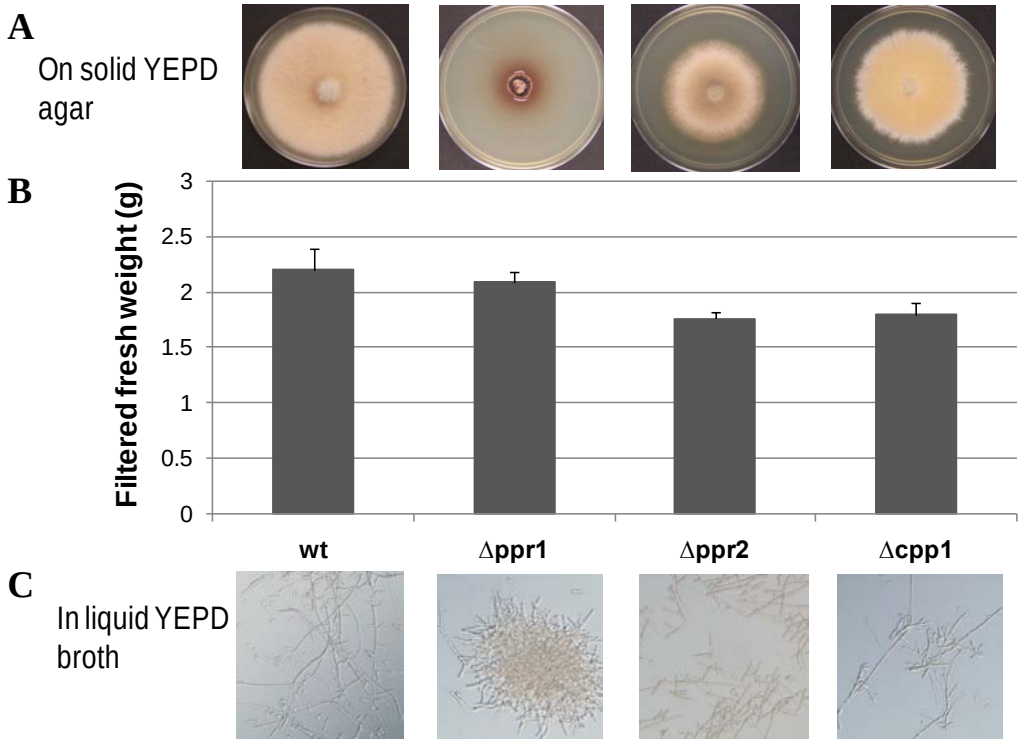

Figure 11. Growth and morphology of the wild type (wt) and mutant strains on YPED agar and in YEPD broth. (A) Growth on solid YEPD agar of wild-type (wt), Δppr1, Δppr2, and Δcpp1 (from the left); (B) filtered fresh weight measured and presented as a bar graph. 10^6 spores of each strain were collected and inoculated into YEPD broth, and incubated for 7 days. Results are means ± *sd* (standard deviation) of three biological replications; (C) microscopic observation of the wild type (wt), Δppr1, Δppr2, and Δcpp1 (from the left) after 7 days incubation.

3.5 Δppr2 strain produces higher level of FB_1 similar to Δcpp1 strain

The level of FB_1 produced on cracked corn medium (Shim and Woloshuk, 1999) was examined by HPLC in wild type, Δppr1, Δppr2, and Δcpp1 to study how disruption of each subunit of PP2A affects FB_1 biosynthesis in *F. verticillioides*. As shown in previous study by Choi and Shim (2008b), Δcpp1 produced about 10 times higher level of FB_1 than the wild type (Fig. 12A). Here, I found that FB_1 production in Δppr1 and Δppr2 differ from that of wild type, but in opposite manner. Δppr2 showed significant up-regulation of FB_1 synthesis compared to that of wild type (Fig. 12A) whereas Δppr1 produced significantly less FB_1 than the wild type.

Subsequently, I performed ergosterol analysis to determine the growth of fungal strain in corn kernels, and this was performed as an effort to standardize FB_1 production per fungal mass in cracked corn media. Figure 12B shows the mean ergosterol production with four replications, and ANOVA (Analysis Of Variance between groups) test confirmed that each mean value is statically different ($P<0.001$). Subsequently, the mean FB_1 level of each strain was divided by the mean of ergosterol from each strain (Fig. 12B). I determined that the FB_1/ ergosterol ratio in Δppr2 is 10 times greater than that of the wild type (Fig. 13) whereas Δppr1 produced approximately 50% level of FB_1 when compared to the wild type.

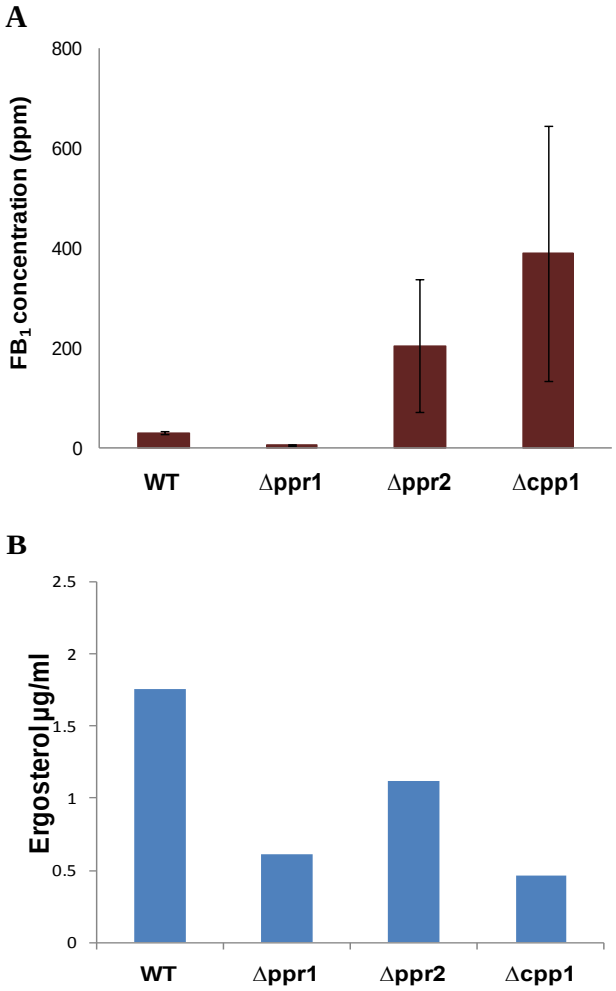

Figure 12. FB_1 and ergosterol analyses by HPLC. Quantification of FB_1 production (A) and ergosterol production (B) in the wild type (WT), Δppr1, Δppr2, and Δcpp1 strain. 10^5 spores of each strain were inoculated to sterile cracked corn (2g) and incubated for 14 days. FB_1 concentration was calculated as ppm (µg/ml). Results are means ± *sd* (standard deviation) of three biological replications (A) or four biological replications (B).

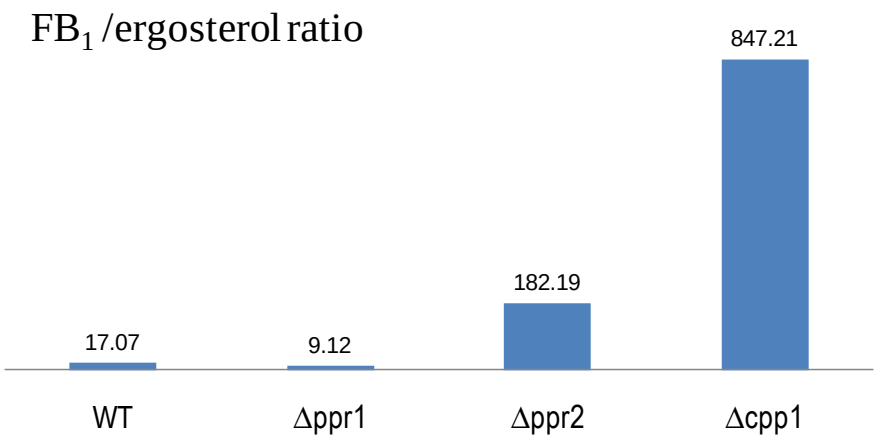

Figure 13. FB_1 production per unit of fungal mass. In the wild type (WT), Δppr1, Δppr2, and Δcpp1 strain, FB_1 /ergosterol ratio (μg FB_1 per μg ergosterol) is calculated as the mean FB_1 production divided by the mean ergosterol in each strain.

3.6 Δppr1 strain produced more microconidia per unit hyphal growth than the wild-type progenitor

I measured microconidia production in four strains to characterize the function of PP2A regulatory subunits on *F. verticillioides* asexual development. After growing the strains on KCl agar for 7 days, I harvested conidia with 0.1% Triton-X solution (2 ml/plate). However, I recognized that due to differences in hyphal growth a more representative assessment approach was needed. Therefore, I calculated the number of conidia on each plate by adjusting the number of conidia with the radial growth area on the plate (Fig. 14).

Surprisingly, Δppr1 produced approximately 3 to 4 times more conidia per unit area than the wild type. This is contradictory to its severe growth defect on solid media (Fig. 9A). On the other hand, Δppr2 produced significantly less conidia than the wild type. I also determined that there was no significant difference in the number of conidia per radial growth between Δcpp1 and the wild type (Fig. 14). These data suggest that PP2A regulatory subunits Ppr1 and Ppr2 play negative and positive roles, respectively, in asexual development independent of the catalytic subunit in *F. verticillioides*.

I also tested sexual reproduction in mutant strains. *F. verticillioides* 7598 (Fungal Genetic Stock Center) was used as the opposite mating-type wild-type strain in sexual crosses, and all mutant strains successfully produced perithecia and viable ascospores, suggesting PP2A subunits are not involved in *F. verticillioides* sexual reproduction.

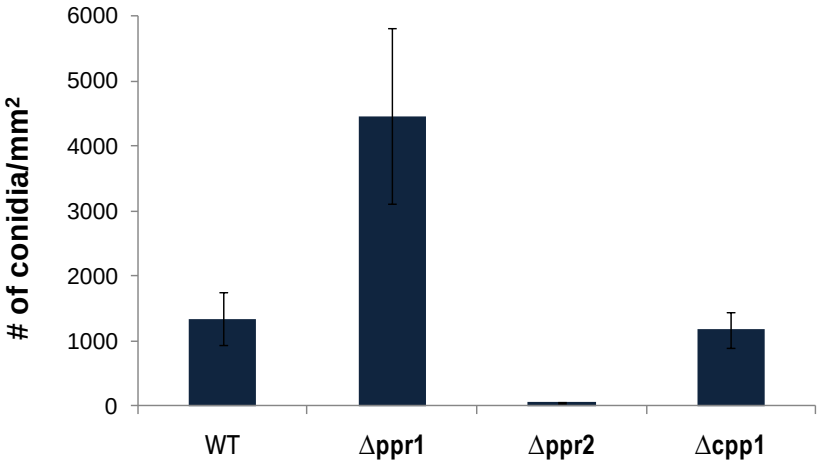

Figure 14. Microconidia production with radial growth. Four strains, the wild type (WT), Δppr1, Δppr2, and Δcpp1, were point inoculated on KCl agar plates with an agar block (0.5 cm diameter), and incubated for 7 days at 25˚C. Conidia were harvested, and quantified with a haemocytometer. The area of radial growth were measured and converted to mm^2. Since the mean area of all wild type and mutant strains were statistically different, the number of conidia were divided by the mean area (mm^2) of radial growth. Three biological replications were performed to obtain standard deviations.

3.7 Disruption of PP2A regulatory subunits show distinct morphological characteristics during conidia germination

Microconidia of *F. verticillioides* wild type strain are single-celled and oval shaped spores (Species Descriptions, 2007). Typically, a microconidium germinates from one tip, and the germ tube establishes hyphal polarity (Fig. 15A). In the PP2A regulatory subunits mutants, I frequently observed abnormal conidia germination (Fig. 15D to 15G). About 20 to 30% of Δppr1 conidia became swollen, and while conidia maintained germ tube polarity, central septation in the cell was observed before germination (Fig. 15D and 15E). I also observed aberrant hyphal swelling when microconidia were germinated (Fig. 15E). This is not as drastic as what Choi and Shim (2008b) observed in Δcpp1 strain, but it suggests that *PPR1* and *CPP1* may serve as regulators of proper hyphal development and structural maintenance. In Δppr2 strain, approximately 10 to 20% of conidia showed Y-shaped early hyphal germination (Fig. 15F and 15G). Typically, an *F. verticillioides* conidium germinates from one tip, and after establishment of the primary germ tube, a secondary germ tube emerges from the opposite tip. I asked whether this abnormal Y-shaped germination is due to simultaneous germination from one tip or early branching from the primary germ tube, and to answer this question I performed time-lapse microscopy to monitor spore germination with 20 minute intervals. In Fig. 16, I observed branch formation in the primary germ tube shortly after germ tubes from each opposite ends, while stunted, were established. This

result suggests that *PPR2* plays a key role in conidial germination preventing early hyphal branching in *F. verticillioides*.

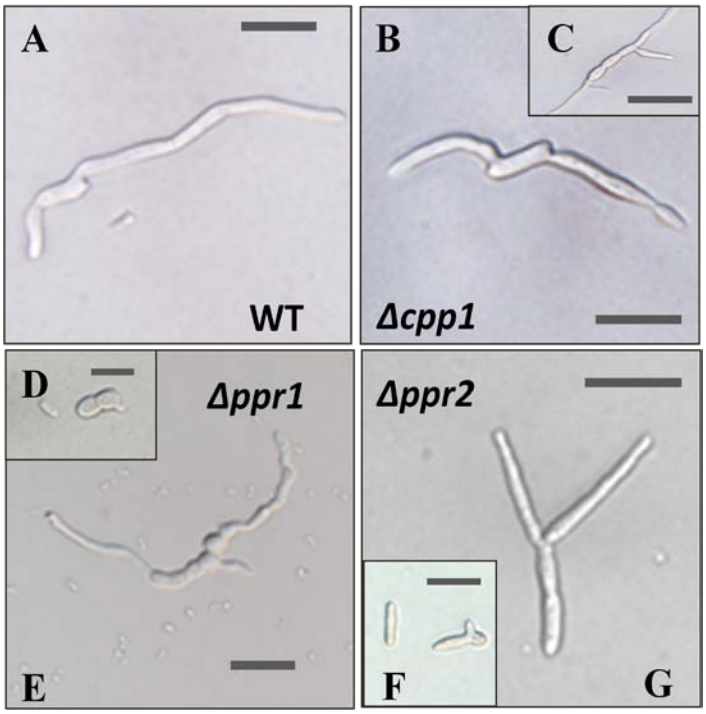

Figure 15. Abnormal morphologies in conidial germination. Germinating conidia of the wild type (WT), Δcpp1, Δppr1, and Δppr2 grown in YEPD liquid media were observed. (A) germination of wild type microconidia; (B) microconidia of Δcpp1; (C) macroconidia of Δcpp1; (D) swollen and central septated Δppr1 microconidia; (E) further developed Δppr1 hypha showing short, swollen, and multi-septated morphology; (F) microconidia of Δppr2; (G) germinated Δppr2 microconidia clearly showing y-shaped branch morphology. Each strain was incubated for 15hrs at 25 ˚C before observation under compound microscope. Scale bar = 10 μm (in A-B, D, F-G). Scale bar=50 μm (in C and E).

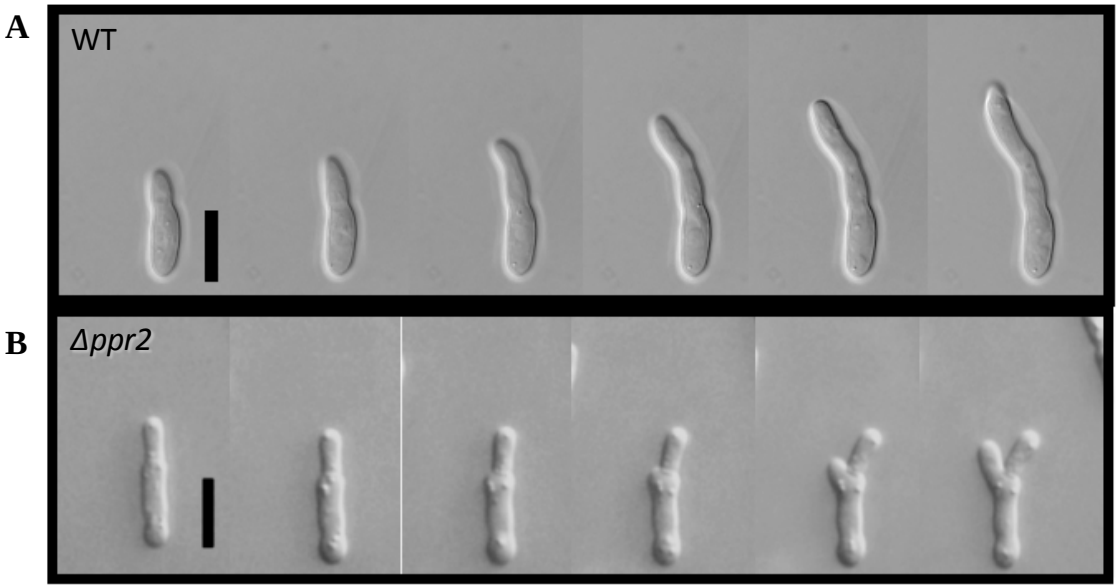

Figure 16. Time-lapse monitoring of wild-type and Δppr2 conidial germination. The first panel demonstrates the first detectable emergence of the germ tube. Each subsequent panel represents exactly 20 min after the panel to its left. (A) Upper panel = wild-type (WT) (Sagaram and Shim, 2007). (B) Lower panel = Δppr2. Scale bar = 10 μm.

4. DISCUSSION

4.1 PP2A catalytic and regulatory subunits in *F. verticillioides*

I identified two PP2A regulatory genes, *PPR1* and *PPR2*, in *F. verticillioides*. Two types of PP2A regulatory subunits, B and B', have been characterized in budding yeast, Cdc55p and Rts1p, respectively. High percentages of protein sequence identities suggested that Ppr1and Ppr2 conform to Cdc55p and Rts1p, respectively. Holoenzyme formation of PP2A subunits is necessary for the multifunctional PP2A complex to perform precise and specific activities in certain developmental stages in eukaryotes (Goldberg, 1999; Mayer-Jaekel and Hemmings, 1994; Virshup, 2000). Therefore, identifying the role of each subunit can provide fundamental understanding of how PP2A regulates important cellular functions. B-type regulatory subunits have been characterized in *N. crassa* and *S. sclerotiorum*, however, generating a *PPR2* deletion mutant in *F. verticillioides* is noteworthy because characterization of the regulatory B' subunit has not been reported in filamentous fungi (Erental et al., 2007; Yatzkan and Yarden, 1999). In this study, I generated and compared three mutants, Δcpp1, Δppr1, and Δppr2, with the goal of elucidating the role of PP2A in regulation of cellular functions in *F. verticillioides*, and perhaps in filamentous fungi.

4.2 Growth regulation by PP2A in *F. verticillioides*

Deletions of PP2A regulatory subunits and the catalytic subunit resulted in significantly diverse phenotypes, supporting the idea that each PP2A subunit is associated with different roles. Among those subunits, it is plausible that *PPR1*, which encodes the PP2A regulatory B subunit, plays a major role in hyphal growth in *F. verticillioides*, as deduced from severe radial growth defects observed in Δppr1 strain. A similar result was observed when *RGB-1* was mutated in *N. crassa* – Ppr1 is homologous to Rgb-1(Yatzkan and Yarden, 1999). However, in addition to slow colony growth, I observed significantly stout cell compartments of the fungus throughout the Δppr1 culture. Yellman and Burke (2006) showed that Cdc55, a homolog of Ppr1, was required for holding spindle checkpoint during mitosis in budding yeast. Thus, severe growth defect accompanied by stout cell compartments in Δppr1 lead us to hypothesize that Ppr1 plays an important role in cell cycle regulation in *F. verticillioides*.

Interestingly, there was no statistically significant difference in the biomass of Δppr1 and wild-type strains when they were cultured in YEPD broth. This was unexpected when considering how Δppr1 grew on solid media. However, I did observe a number of compact hyphal clusters in Δppr1 culture, and it would be reasonable to argue that Δppr1 growth defect observed in solid agar did not hamper fungal mass production in shaking, liquid culture.

In contrast to the Δppr1 growth, Δcpp1 has a less pronounced growth defect in all media tested. I can think of two hypotheses to explain how a regulatory subunit can have

more drastic impact on growth. First, there is a possibility that other putative proteins, perhaps different phosphatase complexes, can help the fungus bypass PP2A defect. For instance, in budding yeast, triple deletion of *PPH* genes was lethal, but the fact that the triple deletion mutant was viable in high glucose conditions suggests that alternative protein rescued the triple mutation (Hu and Ronne, 1994). In addition, this study showed that overexpression of *PAM1*, the PP2A multicopy suppressor, increased survival rate of the *PPH* triple deletion mutant in lower glucose concentration. However, if this were true, then it would be reasonable to predict that both Δppr1 and Δcpp1 mutants showed share a similar phenotype, rather than the milder defect observed in Δcpp1. Second, I can propose that there are alternative PP2A catalytic subunit(s) that can substitute for Cpp1 and maintain a certain level of PP2A complex function. This could be a more likely possibility since in yeast and *A. nidulans* two genes encoding PP2A catalytic subunit were identified (Kosmidou et al., 2001; Sneddon et al., 1990). Furthermore, in mammalian systems, more than two isoforms of PP2A catalytic subunit can commonly be identified (Janssens and Goris, 2001).

Growth defects observed in Δppr2 also implied that the regulatory B' subunit is involved in hyphal growth in *F. verticillioides*. In yeast, slower and smaller cell growth in B' subunit mutant strain was repeatedly observed, which led to the suggestion that PP2A regulatory B' subunit is associated with stress-related response, regulation of cell cycle progression, septum positioning, and cell-size control (Shu et al., 1997; Tanabe et al., 2001). One other interesting aspect of the regulatory B' subunit is that it can suppress the activity and substrate specificity of the AC dimer, which represents PP2A catalytic C

subunit with scaffolding A subunit (Usui et al., 1988). Therefore, the severe defective growth in Δppr1 could be a result of the loss of balance between B and B' regulatory subunits. In fission yeast, the B subunit over-expression strain exhibited phenotypes that partially overlapped with B' deletion strain, and B' over-expression strain shared the abnormalities shown in the B deletion strain (Kinoshita et al., 1996; Tanabe et al., 2001). However, inhibitory activity of PP2A regulatory B' subunit was not demonstrated in filamentous fungi. Investigation of how, or if, *PPR1* and *PPR2* over-expression influences the cell compartment may provide us a better understanding of how PP2A functions are controlled in *F. verticillioides* and perhaps in other filamentous fungi.

4.3 PP2A regulation in FB_1 and secondary metabolites

The results suggest that Ppr2 is the regulatory subunit that influences the PP2A complex to regulate FB_1 synthesis in *F. verticillioides*. In budding yeast, deletion of *RTS1* resulted in constitutive expressions of amino acid permeases (AAPs) which are normally activated when uptake of amino acids is needed (Eckert-Boulet et al., 2006). Since FB_1 production in *F. verticillioides* can be facilitated under nitrogen stress condition (Shim and Woloshuk, 1999), I can propose that the AB'C form of PP2A is involved in recognition of a nitrogen signal pathway in *F. verticillioides* whereas the ABC form functions in an opposite manner. Furthermore, as seen in Fig. 10, Δppr1 produced heavy pigmentation, suggesting that PP2A negatively regulates a majority of secondary metabolites. Secondary metabolites are often produced by clustered genes,

like *FUM* gene cluster, and are activated by regulatory transcription factors (Yu and Keller, 2005). It can be presumed that Ppr1 activity may be required upstream of these transcription factors to prevent unnecessary production of secondary metabolites. However, since I did not identify the specific secondary metabolites in the pigment of Δppr1, it is difficult to further speculate how PP2A is involved in the regulation of various secondary metabolites in *F. verticillioides*.

4.4 PP2A regulation in conidiation and germination in *F. verticillioides*

While macroconidia production was one of the striking phenotypes in Δcpp1, which suggested that PP2A catalytic subunit is involved in the regulation of macroconidia-microconidia equilibrium (Choi and Shim, 2008b), macroconidia were not observed in Δppr1 or Δppr2 strains. Involvement of PP2A regulatory B subunit in macroconidia production has been previously discussed in *rgb-1* mutation in *N. crassa* (Yatzkan and Yarden, 1999). However, our result suggests that the PP2A catalytic subunit is the key player in macroconidia-microconidia equilibrium in *F. verticillioides*. It is also important to note this result does not lead to a firm conclusion that Ppr1 and Ppr2 are not directly associated with sexual spore development. One of the difficulties associated with testing macroconidiation in *F. verticillioides* is that I do not have established protocols to consistently produce macroconidia under laboratory condition.

Microconidia morphology and germination pattern in the three mutant strains were significantly different. Overproduction of microconidia in Δppr1 resembles the

increased arthroconidia production in *rgb-1* mutant. Rgb-1 is required for major constriction formation in *N. crass*a macroconidiation pathway (Yatzkan and Yarden, 1999). Microconidia overproduction was also observed when *FvVE1*, the homolog *VeA* of *A. nidulans*, was deleted in *F. verticillioides* (Li et al., 2006). ΔFvve1 also produced microconidia, which is similar to one of the phenotypes of Δcpp1. Therefore, I can assume that Cpp1 and Ppr1 are downstream of Fvve1 but perform different functions. However, the genetic link between *FvVE1* and *CPP1* has not been determined to date (Choi and Shim, 2008b).

About 10 to 20% of germinating conidia of Δppr1 exhibited abnormal morphology, notably central septation. Typically, septation is a product of cell division in filamentous fungi. In fission yeast, during mitotic cell division, Plo1 kinase accumulates, which leads to increased kinase activity (Tanaka et al., 2001). Considering the important role of PP2A regulatory B subunit in mitosis, I can anticipate Ppr1 involved in the regulation in Plo1-like kinase activity. However, it is unclear whether the ABC heterotrimer of PP2A directly controls the factors inducing septation. Septation in germinating Δppr2 strain was normal, and this result disagrees with the multi-septation phenotype of the B' subunit deletion mutant in fission yeast (Tanabe et al., 2001). Interestingly, conidia of Δppr2 developed a new hyphal branch just after germination. According to the review by Harris (2008), this branching pattern is similar to the lateral branching pattern which is generally associated with septation and/or newly formed spitzenkorper. In Δppr2, early-branching was observed only in the axillary of the primarily germinated tip, suggesting a certain orientation change in tip growth. Further

observations of microtubule network associated with generating a hyphal tip will be required to investigate the detailed role of Ppr2 in branching during early germination in *F. verticillioides*.

4.5 Localization of three PP2A subunits

In this study, I isolated three PP2A subunits in *F. verticillioides*, and functionally analyze them by independently generating deletion mutants. To figure out the detailed mechanisms of PP2A involvement in various cellular functions, further investigations of these three subunits at the protein level will be required. Particularly, determining the localization of two PP2A regulatory subunits could be the important first step. While the complete PP2A complex is essential for substrate specificity and proper function, localization of each regulatory subunit can help us understand how and where PP2A functions are performed. The dynamic localization pattern of each subunit was monitored in *S. cerevisiae* (Gentry and Hallberg, 2002), and interestingly, Cdc55p and Rts1p, two PP2A regulatory B subunits, localized to different sites during mitosis. Likewise, since I observed significantly different germinating phenotypes in Δppr1 and Δppr2, characterizing the localization of these PP2A subunits during germination will help us better understand how specific activities of PP2A regulatory subunits are achieved in *F. verticillioides*.

5. SUMMARY

Two PP2A regulatory subunits, Ppr1 and Ppr2, which are homologs of Cdc55p and Rts1p in *S. cerevisiae*, respectively, were identified in *F. verticillioides*. The *ppr1* deletion mutant (Δppr1) showed severe hyphal growth defect when growing on solid media and swollen and shorter cell compartment morphology under a microscope, suggesting that Ppr1 is involved in microtubule distribution and cell cycle control. Over-production of microconidia and heavy pigment accumulation were also observed in Δppr1 strain suggesting that Ppr1 negatively regulates signaling pathways triggering various secondary metabolites and asexual reproduction in *F. verticillioides*. Interestingly, Δppr2 strain showed up-regulation of fumonisin production whereas Δppr1 produced lower level of fumonisin than the wild-type progenitor, suggesting unique, but contradictory, role of PP2A regulatory subunits in the regulation of fumonisin biosynthesis. Both deletion mutants exhibited distinct morphological abnormality during conidia germination: swelling and central septation during germination in Δppr1 and early branching in Δppr2. Results from this study suggest that two PP2A regulatory subunits carry out critical roles in regulating fumonisin biosynthesis and fungal development in *F. verticillioides*.

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